

A strong B cell response is part of the immune landscape in human high-grade serous ovarian metastases.

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Translational relevance:

Little is known about how B cells interact with other players of the tumor immune landscape in HGSOC, especially in the context of a metastatic disease. The present study provides evidence for a positive role of B cells in the development of an anti-tumor immune response at metastatic sites; an effect that can be enhanced by chemotherapy treatment. A better understanding of these processes is essential for developing new anti-cancer immunotherapy strategies. We show that it would be beneficial to take advantage of B cells and their immunoglobulins to boost the anti-tumor immune response in patients with advanced stage HGSOC, in particular to increase DC recruitment to TLS and their antigen presenting ability. This could be exploited in DC-based vaccination protocols or as adjuvant to different anti-cancer treatments.

Abstract

Purpose

In high-grade serous ovarian cancer (HGSOC), higher densities of both B cells and the CD8⁺ T cell infiltrate were associated with a better prognosis. However, the precise role of B cells in the anti-tumor response remains unknown. As peritoneal metastases are often responsible for relapse, our aim was to characterize the role of B cells in the anti-tumor immune response in HGSOC metastases.

Experimental design

Unmatched pre and post-chemotherapy HGSOC metastases were studied. B-cell localization was assessed by immunostaining. Their cytokines and chemokines were measured by multiplex assay and their phenotype was assessed by flow cytometry. Further *in vitro* and *in vivo* assays highlighted the role of B cells and plasma cell IgGs in the development of cytotoxic responses and dendritic cell activation.

Results

B cells mainly infiltrated lymphoid structures in the stroma of HGSOC metastases. There was a strong B-cell memory response directed at a restricted repertoire of antigens and production of tumor-specific IgGs by plasma cells. These responses were enhanced by chemotherapy. Interestingly, transcript levels of CD20 correlated with markers of immune cytolytic responses and immune complexes with tumor-derived IgGs stimulated the expression of the co-stimulatory molecule CD86 on antigen presenting cells. A positive role for B cells in the anti-tumor response was also supported by B cell depletion in a syngeneic mouse model of peritoneal metastasis.

Conclusions

Our data showed that B cells infiltrating HGSOC omental metastases support the development of an anti-tumor response.

Introduction

The immune system can both limit and promote cancer development. Immune cells infiltrate tumors and recent trials showed how unleashing a tumor-specific immune response with the use of tumor vaccines or immune checkpoint blockade can constitute a successful cancer therapy (1,2). The majority of cancer immunology studies have concentrated on the pro or anti-tumor abilities of T cells or myeloid cells. Less is known about the role of B cells in the tumor microenvironment, especially their contribution to the metastatic niche. In pre-clinical models of melanoma, squamous cell carcinoma and carcinogen-induced skin cancer, B cells promote tumor progression through the production of immune regulatory cytokines and immune complexes (3-5). On the other hand, in human primary tumors, the presence of B cells in association with tertiary lymphoid structures (TLS) in non-small cell lung carcinoma (NSCLC), colorectal, ovarian and pancreatic cancer has been associated with a better prognosis (6-9). In these tumors, the presence of both B cells and dendritic cells (DCs) correlated with an increase in Th1 signature, which might explain the correlation with better survival.

Very few studies have described the immune landscape of human metastases. Lymphoid structures were identified in cutaneous metastases of melanoma patients (10) as well as in lung metastases of colorectal (CRC) and renal cell carcinoma (RCC) patients (11). Interestingly, a high infiltration of CD8⁺ T cells and DC-LAMP⁺ DCs correlated with an increased overall survival (OS) of patients with CRC whereas this correlated with decreased OS of patients with RCC (11). B cells have been described in TLS, however, their role in the tumor immune landscape remains unclear. In primary ovarian cancer biopsies, intra-tumor infiltration of CD27⁺ atypical memory B cells, together with CD8⁺ T cells, is linked to better prognosis (12). A very recent

study also showed that a high infiltrate of T cells, B cells and plasma cells in primary tumors is linked to the presence of TLS in the microenvironment and improved survival of patients (13). Whether B cells behave the same way in ovarian cancer metastases and how they influence the anti-tumor response is unknown.

The term ovarian cancer refers to a group of five diseases defined as high grade serous, low grade serous, mucinous, endometrial and clear cell carcinomas that are known to arise from different organs and have different molecular and transcriptomic profiles but all spread into the peritoneal cavity (14,15). High-grade serous ovarian cancer (HGSOC) is the most common subtype, representing about 70% of cases and the majority of deaths from ovarian cancer (14). Early detection of the disease is one of the biggest challenges, as most patients are diagnosed at an advanced stage with metastases disseminated in the peritoneal cavity. Platinum-based chemotherapy and surgical de-bulking represent the baseline treatment for HGSOC and can prolong survival, although the majority of patients eventually relapse and die of peritoneal disease. Therefore, understanding the biological properties of the peritoneal metastases and their immune infiltrate is essential to develop new treatment strategies that target the tumor deposits responsible for relapse.

In order to elucidate the role of B cells in omental metastasis from HGSOC patients, we analyzed ninety-two omental samples obtained after surgery. B cells were mainly located in lymphoid aggregates, which displayed characteristic features of TLS. The majority of B cells had a memory phenotype, displayed a restricted clonal repertoire compared to peripheral healthy B cells and produced cytokines and chemokines known to recruit and activate anti-tumor immune cells, such as dendritic cells, T cells and NK cells. Using RNAseq analyses and *in vivo* experiments we also showed that B cells are implicated in immune cytotoxic responses.

Furthermore, B cells differentiated to plasma cells and produced immunoglobulins (Igs) against tumor targets. Igs can be found bound to antigen-presenting cells (APC) in the tumor stroma and co-culture of APCs with immune complexes (ICs) generated *in vitro* with tumor IgGs increased the expression of the co-stimulatory molecule CD86, suggesting that Igs might promote an anti-tumor response through potentiation of DC priming. Overall, our data suggest that B cells favor the development of an anti-tumor immune response in omental metastasis of HGSOC.

Materials and Methods

Patient samples. Omental tissues were collected from high-grade serous ovarian cancer patients (FIGO stage III and IV) prior or following platinum-based chemotherapy treatment (Supplementary Table S1). Samples were either formalin fixed and paraffin embedded or snap frozen and stored at -80°C or processed as fresh tissues. Blood samples from pre and post-chemotherapy patients were collected in sodium heparin tubes for plasma separation and EDTA tubes for cell analysis. Control blood was collected from 12 healthy volunteers. Institutional review board approval was granted for the Barts Gynae Tissue Bank to collect and store biological material and clinical information. Patients were treated at St Bartholomew's Cancer Centre between 2010 and 2016 and gave written informed consent. Clinical parameters were collected using tissue repository databases and chart review.

Cell lines. The AOCS1 cell line was kindly given by Professor David Bowtell (Peter MacCallum Cancer Center, Melbourne, Australia) and was established from the ascites of a patient diagnosed with HGSOc, Silverberg Grade 3, after second relapse. The G33 cell line was established in our laboratory from an omental HGSOc tumor collected during interval debulking surgery in 2011 (16). The cell lines have been authenticated. Cells were cultured in DMEM/F12 (Gibco), 10% FCS, 2mM L-glutamine, 1% pen-strep in a 5% CO₂ humidified incubator at 37°C. Regular checks for mycoplasma contamination performed.

Tissue processing and flow cytometry. The stroma vascular fraction (SVF) was isolated from fresh omentum by mechanical dissection of the tissue with a scalpel and collagenase D digestion for 30min at 37°C (Roche). Digested samples were filtered

through 70µm strainers and the SVF were used for FACS analyses. Fresh PBMCs were isolated from blood samples by centrifugation with Ficoll-PaqueTM Plus (GE Healthcare).

Cell suspensions were stained with different antibody panels for 30min on ice in PBS, 2.5% bovine serum albumin and 2mM EDTA. Cells were then washed twice and analyzed directly by FACS or fixed in PBS 2% formalin for 10min at 4°C before being analyzed. Antibodies and viability dyes are shown in supplementary Table S2. Appropriate fluorescence minus one (FMO) controls were used in these experiments. Analysis was performed using an LSR Fortessa cell analyzer (BD-Biosciences).

Immunostaining of FFPE sections. Paraffin-embedded sections from HGSOc omental metastases were stained for CD20, CD4, CD8, IgG, IgM, CD68, Ki67, FDC, Myeloperoxidase (MPO) (Dako), MECA79 (Biolegend) and DC-LAMP (Sigma). Sections were deparaffinised in xylene for 10min and re-hydrated by successive immersion in 100%, 90%, 70%, 50% EtOH and H₂O. Heat induced antigen retrieval was performed with Citrate Buffer (Vector Labs) for 20min in a pressure cooker.

Immunohistochemistry: Slides were blocked in PBS 2.5% BSA and 2.5% goat serum for 40min at room temperature (RT) and biotin/streptavidin blocking buffer (Vector Labs) according to manufacturer's recommendations. Primary and secondary antibodies were applied for 1h at RT. Enzymatic activity was revealed with the Vectastain ABC Elite kit (Standard) (Vector Labs) and Sigma FAST 3,3'-diaminobenzidine tablets (Sigma). Sections were counterstained with hematoxylin and mounted with DPX mounting medium (Sigma).

For dual-stain immunohistochemistry, antibodies were incubated sequentially and revealed with the Vectastain system previously described. FAST 3,3'-

diaminobenzidine tablets were used to generate the brown color and the Impact VIP peroxidase substrate kit (SK-4605 Vector labs) generated the purple color. Sections were mounted with Dako Faramount aqueous mounting medium.

Brightfield pictures we taken with an Axiophot microscope (Zeiss). CD20, DC-LAMP and MPO single stained slides were scanned using a Pannoramic Scanner (3D HISTECH) and analyzed with the “Tissue Studio” software 4.0 (Definiens).

To assess numbers of MPO⁺ neutrophils and DC-LAMP⁺ DCs in lymphoid structures, 2-5 TLS per tumor were analyzed to obtain a mean number of positive cells per TLS for each metastasis. Intra-vessel cells were excluded from the counts.

Immunofluorescence: Slides were incubated for 20 min in NH₄Cl (50mM) to reduce autofluorescence and blocked in PBS 2.5% BSA, 2.5% goat serum for 40min at RT. IgG staining was performed prior to any other additional staining. Subsequently, primary antibodies were applied overnight at 4°C. The next day, fluorescently labeled secondary antibodies (Invitrogen) were incubated for 1h at RT and sections were mounted with Prolong gold antifade reagent containing Dapi (Thermo Fisher Scientific).

The specificity of the stainings was determined using appropriate isotype controls. Fluorescent pictures were taken with an LSM510 confocal microscope (Zeiss).

***In vitro* DC differentiation and activation.** PBMCs were isolated from the blood of 6 healthy volunteers by centrifugation with Ficoll-PaqueTM Plus (GE Healthcare). CD14⁺ monocytes were purified using anti-human CD14 beads (Miltenyi) and cultured in RPMI 10% FCS, 20ng/ml recombinant human GM-CSF and recombinant human IL4 (Biolegend). After 6 days, monocyte-derived DCs were re-stimulated with 10µg/ml of IgGs (purified from protein extracts of two HGSOC omental tumors or

healthy plasma IgGs and pooled) for 24h hours, pre-incubated or not with 30µg of combined protein extracts from HGSOc cell lines, G33 and AOCS1. Expression of the co-stimulation marker CD86 on DCs was assessed by flow cytometry.

Statistical analysis: Data are expressed as the mean of values per sample \pm the standard error of the mean (SEM). Statistical significance for flow cytometry, immunohistochemistry and Mesoscale analyses was assessed using the Student's T test or a non-parametric test (Mann-Whitney) when appropriate. Spearman or Pearson tests were used for correlation analyses. Data normal distribution was assessed using the D'Agostino & Pearson omnibus normality test. Data were considered significant from $p < 0.05$.

For the mouse experiment luminescence intensity values were \log_{10} transformed before applying a one-way ANOVA test to account for heteroscedasticity of results.

For BCR sequencing data analyses, we used the species richness, the Gini-Simpson index and the Shannon entropy as diversity indexes.

Finally, for the RNAseq data analysis, significantly differentially expressed genes were identified using a double threshold of false discovery rate (FDR) < 0.05 and an absolute fold change > 2 .

Results

Presence of B cells in TLS in HGSOC omentum is associated with the generation of memory B cell responses.

To assess the presence and localization of B cells in HGSOC omental metastases, paraffin sections from unmatched biopsies taken pre (n=10) and post (n=31) neoadjuvant chemotherapy (NACT) were stained for the B cell marker CD20 (Supplementary Table S3). CD20⁺ B cells were mainly localized in lymphoid aggregates in the stroma, next to tumor islets (Figure 1A and B). There was no significant difference in the number of B cells in pre and post NACT biopsies, suggesting that chemotherapy neither impairs nor increases B cell infiltration in omental metastases (Figure 1C). Because similar lymphoid structures were described in other solid cancers as TLS (7,10,17), we further characterized the B cell rich follicles using immunohistochemistry and immunofluorescence. We often observed a core of CD20⁺ B cells surrounded by a T-cell rich area of mainly CD4⁺ T cells, whereas CD8⁺ T cells were located at the periphery of the lymphoid aggregates (Figure 1D). In addition, high endothelial venules (HEV; blood vessels specialized in the recruitment of immune cells within lymphoid organs) were present, as indicated by MECA79 staining (Figure 1E). By double color immunohistochemistry we also identified areas rich in follicular dendritic cells and Ki67⁺ highly proliferative germinal center B cells surrounded by CD45RO⁺ memory cells (Figure 1F). Taken together, these features indicate the presence of TLS in HGSOC omental metastases.

TLS are implicated in the differentiation of antigen specific B cells and development of memory responses (8). To assess the B cell composition of HGSOC omental metastases, flow cytometry analysis of cells extracted from 25 fresh HGSOC omenta was carried out. Results showed that a majority of omental B cells display a

“classical” ($CD27^+IgM^+$ and $CD27^+IgM^-$) or “atypical” ($CD27^-IgM^-$) memory phenotype (Figure 2A and B). Interestingly, we observed a significant increase in the proportion of class-switched memory B cells ($CD27^+IgM^-$) in metastases from NACT-treated patients compared to pre-chemotherapy patients (Supplementary Figure S1A). This was accompanied by a decrease in $CD20^+$ naïve B cells after chemotherapy treatment, although data did not reach statistical significance. The extent of residual disease did not seem to impact significantly on the proportion of class-switched memory B cells after chemotherapy (Supplementary Figure S1A).

The percentage of naïve B cells was on average 28.2% of $CD20^+$ cells in omental metastasis (Figure 2B), lower than in patient blood (mean 47.8%; Supplementary Figure S1B). This difference suggested that there was an ongoing B cell response in HGSOC patients that drove B cell differentiation and might have taken place in TLS at sites of metastasis.

Further analyses of B cell subpopulations demonstrated that a higher proportion of class-switched memory B cells ($CD20^+CD27^+IgM^-$ and $CD20^+CD27^-IgM^-$) expressed the co-stimulatory molecule CD86 when compared to IgM^+ memory ($CD27^+IgM^+$) and naïve ($CD27^-IgM^+$) B cells (Figure 2C). No difference in CD86 expression was observed between pre and post chemotherapy patients (Supplementary Figure S1C). Nonetheless, all memory B cell subpopulations also upregulated the inhibitory receptor PD1, albeit to a lesser extent (Figure 2D and Supplementary Figure S1D). Co-expression of CD86 and PD1 on B cells was generally low (Supplementary Figure S1E and F).

To investigate whether omental B cells were mounting an antigen specific response, we then analyzed their clonal repertoire by B cell receptor (BCR) sequencing. We extracted RNA from purified omental HGSOC B cells and peripheral B cells from

healthy individuals and sequenced the *IgH* portion of the B cell receptor. We report here three common mathematical indices of diversity calculated from the *IgH* repertoire data: the species richness (defined as the number of unique *IgH* clonotypes in the sample), the Shannon entropy (which equally weights a species richness term and a term that increases with increasing clonal dominance), and the Gini-Simpson index (which weights the dominance term more heavily than the species richness term). For all diversity indices, HGSOC B cells showed decreased diversity relative to healthy donor B cells (Figure 2E). This observation was in accordance with an increase in the rate of somatic hypermutations in Igs from HGSOC omentum compared to healthy B cells (Figure 2F).

So far, our results suggested that B cells in HGSOC omentum are activated, differentiate to a memory B cell phenotype and undergo clonal selection.

B cells correlate with the infiltration of DC-LAMP⁺ DCs and an immune cytolytic signature in HGSOC omentum.

B cells secrete pro-inflammatory and/or regulatory cytokines and chemokines that can influence the development of specific immune responses, as previously found in different cancer models (18,19). To determine how the secretome of HGSOC B cells could influence the anti-tumor immune response, B cells from omental metastases were purified and stimulated for 48h with a low dose of PMA and Ionomycin (PMA/Iono). The secretion of cytokines and chemokines was analyzed by electrochemoluminescence multiplex assay and healthy peripheral B cells were used as control.

The heatmap shown in Figure 3A highlights the relationship existing between 24 cytokines and chemokines produced by HGSOC omental B cells and suggests an

immune-stimulatory role for B cells. In addition to secretion of cytokines and chemokines (GM-CSF, IFN γ , IL12p40, CXCL10, IL7) that can broadly recruit and support macrophages, DCs, T cells and NK cells, tumor omental B cells produced significant amounts of inflammatory cytokines such as CXCL8, CCL2 and IL6, even in the absence of PMA/Ionomycin stimulation (Figure 3B-D). Median concentrations were 19694 pg/ml for CXCL8, 885.5 pg/ml for CCL2 and 51.4 pg/ml for IL6, as opposed to 2178 pg/ml, 2.19 pg/ml and 2.027 pg/ml respectively in HGSOC and healthy peripheral B cells.

When we analyzed the expression of the CXCL8 receptors, CXCR1 and CXCR2, on immune cells from HGSOC omentum we observed that, in accordance with previously published evidence (20), granulocytes expressed the highest levels of both receptors, whereas DCs expressed significant but lower levels of CXCR1 and CXCR2 (Figure 3E). B and T cells had very low expression of these two chemokine receptors. These results suggest that through the production of CXCL8, B cells are implicated in the recruitment of both DCs and granulocytes in omental metastases and more specifically in TLS. Given that the majority of granulocytes in HGSOC omentum are neutrophils (data not shown), we stained HGSOC omental tumors for the neutrophil marker MPO (myeloperoxidase) and DC marker DC-LAMP and assessed the localization of DCs and neutrophils in the tumor microenvironment (Figure 3F). Three times less neutrophils than DCs were observed in TLS. We next studied whether there was a correlation between the number of CD20⁺ B cells and DC-LAMP⁺ DCs in HGSOC omentum. Immunohistochemistry staining and digital analysis of 16 FFPE sections from HGSOC omental metastases showed a significant correlation between the density of B cells and DCs in the stroma area of omental tumors (Figure 3G).

In lung cancer, the presence of B cell rich TLS and DCs has been linked to good prognosis and the development of a Th1 signature (21). Since activated DCs can stimulate CD8⁺ T cells and promote a cytotoxic response and B cells and DC densities were correlated in HGSOE metastases (Figure 3G), we assessed whether the B cell infiltrate correlated also with a cytotoxic response. To this end, we analyzed RNA from HGSOE omental metastasis by RNAseq and examined transcriptional changes between uninvolved Stage1/2 HGSOE omentum (n=6), Stage 3/4 untreated (n=4), chemotherapy-treated diseased omentum (bad responders) (n=7) and chemotherapy-treated omentum with low residual disease (good responders) (n=5). When comparing gene expression levels between these groups, forty-four genes, characteristic of a B cell signature, were significantly increased in omental metastases (Stage 3/4 pre and post chemo and Stage 3/4 good responders as opposed to Stage 1/2) (Figure 4A-D). We next analyzed the correlation between the transcript levels of CD20 in stage 3/4 HGSOE omentum and the immune cytotoxic markers, granzyme A (*GZMA*) and perforin (*PRF1*) (22). Not only did we observe a strong correlation between the levels of *CD20* and *GZMA* or *PRF1* transcripts (Pearson $r=0.73$ and 0.61 respectively; data not shown) but *CD20* expression also strongly correlated with the geometric mean of the combined parameters (Figure 4E). This suggested that B cells contribute favorably to the development of an anti-tumor immune response.

The above associations were supported by an experiment using a syngeneic mouse model of ovarian cancer, in which the mouse cell line ID8 is injected intraperitoneally and tumor growth is monitored over the course of 8 weeks. We depleted CD20⁺ B cells in tumor-bearing mice with an anti-CD20 antibody and found that B cell depletion was associated with an increased tumor progression (Figure 4F, left panel) and a reduced T cell cytotoxic activity, as indicated by decreased expression of the

degranulation marker CD107 from ascitic CD8⁺ T cells (Figure 4F, lower right panel).

Collectively our results suggest that through the production of cytokines and chemokines that help recruit and support antigen-presenting cells, B cells could enhance the development of an immune cytotoxic response towards tumor cells.

Immunoglobulin production by intratumor plasma cells

Active germinal center reactions in TLS are normally associated with differentiation of plasma cells that secrete antigen-specific immunoglobulins. Indeed, HGSOC omenta appeared to contain a plasma cell infiltrate, as demonstrated by the presence of CD38⁺⁺CD19^{+/-}CD20⁻CD27⁺ cells (Figure 5A and 5B). Furthermore, we observed high levels of IgG deposits in HGSOC omenta, mainly located in stromal areas but also bound to tumor cells and macrophages (Figure 5C). IgM immunoglobulins were also detected but to a lesser extent (Supplementary Figure S2A). Mass spectrometry analysis of proteins extracted from three HGSOC omental metastases confirmed that IgG immunoglobulins represent the largest subclass, with IgM and IgA representing less than 15% of total antibodies. IgE immunoglobulins were not detected under these conditions (Supplementary Figure S2B). We next examined the amount of IgG1, IgG2, IgG3 and IgG4 subclasses in HGSOC omenta from different disease Stages (1/2 vs 3/4) with high or low residual disease (Supplementary Table S4). Figure 5D shows an increase in the presence of IgG1, IgG2 and IgG3 in stage 3/4 omenta compared to Stages 1/2 (Figure 5D and Supplementary Figure S2C). Furthermore, IgG3 was significantly increased in post-chemotherapy diseased samples compared to pre-treatment samples. On the other hand, IgG4 levels seemed to increase in Stage 3/4 pre-chemotherapy tumors compared to Stage 1/2 uninvolved omenta but had a

tendency to decrease post-chemotherapy. Importantly, HGSOC patient plasma levels of IgG subclasses were not different from healthy controls (Supplementary Table S5 and Supplementary Figure S2D and E), suggesting that a tumor-specific antibody response preferentially takes place at the tumor site in TLS.

Next, to assess the ability of omental IgGs to recognize membrane bound and/or intracellular tumor antigens, we isolated immunoglobulins from three HGSOC omental tumors (G146, G168 and G175) and tested their reactivity towards intact or permeabilized tumor cells (AOCS1 and G33 HGSOC cell lines) by flow cytometry and immunofluorescence respectively (Supplementary Figure S3A-C). IgGs from all three omenta mainly targeted intracellular antigens; either cytoplasmic or nuclear (Supplementary Figure S3A and B), nonetheless, IgGs extracted from the G175 and G146 omental tumors were able to recognize antigens expressed at the surface of a subpopulation of the HGSOC tumor cell line AOCS1 (Supplementary Figure S3C).

To determine the nature of these antigens we carried out immunoprecipitation experiments using purified tumor immunoglobulins as bait and assessed proteins pulled down by mass spectrometry. Total tumor lysate and control bead extracts were used as controls. Proteins found enriched in the IP fraction (but not in the control bead extracts) compared to the total tumor lysate were considered potential targets of intra-tumor IgGs (Table 1). This assay identified a number of targets of omental IgGs, some of which were validated by IHC. The proteins GNA13 (Guanine nucleotide binding protein (G) 13), DPYSL3 (Dihydropyrimidase like 3 or CRMP4) and HSPB1 (heat shock protein B1) were detected in paraffin sections of HGSOC omenta (Supplementary Figure S3D). We concluded from these experiments that the majority of immunoglobulins were binding antigens released in the tumor microenvironment after tumor cell death. Nevertheless, the extensive deposition of immunoglobulins

bound to antigens, called immune complexes (ICs) could still be playing a role in the anti-tumor response, since ICs can activate APCs via ligation of Fc gamma receptors (FcγR) (23,24). Therefore, we assessed whether purified intra-tumor IgGs were playing a role in the activation of APCs *in vitro*. In these experiments we treated monocyte-derived DCs with tumor-derived IgGs, pre-incubated or not with protein extracts from HGSOc cell lines in order to generate ICs. IgGs from healthy blood, alone or incubated with tumor antigens, were used as controls (Figure 5E). Treatment of monocyte-derived DCs with *in vitro* generated tumor ICs significantly increased the expression of the co-stimulatory molecule CD86 compared to healthy IgGs in combination with tumor antigens. This suggested that IgGs, likely produced by intra-tumor plasma cells, recognize tumor antigens and form ICs that contribute to the activation of APCs in the tumor microenvironment.

Discussion

In this study we set out to elucidate the role played by B cells in HGSOC metastases by analyzing human biopsies. From previous studies, it was known that high levels of CD8⁺ T cell infiltration of primary human ovarian tumors were associated with an increased survival (25) and that patients displaying high tumor-infiltrating T cells, CD20⁺ B cells, and plasma cells exhibited an additional survival advantage (12,13). This was reinforced by analyses of the TCGA ovarian cancer dataset, showing a positive correlation between a B cell gene expression signature and progression free survival in immunoreactive ovarian tumors (26). However, data were limited to primary tumors and not peritoneal metastases, which are generally responsible for the high rate of recurrence in HGSOC. In our work on omental metastases, we found B cells in HGSOC omentum were mainly located in lymphoid structures and a high proportion exhibited a memory phenotype. These results were in accordance with the above-mentioned study demonstrating memory B cells in primary ovarian tumors, which correlated with a good prognosis (12). Furthermore, a similar role for B cells was also described in NSCLC (8). We do not have sufficient samples to assess correlations with survival, nonetheless our results indicate that B cells take part and positively influence the development of an anti-tumor response. This is in agreement with a pre-clinical study showing that depletion of B cells using anti-CD20 antibodies in the B16 melanoma model impairs CD8⁺ T cell anti-tumor immunity and promotes cancer development (27). Interestingly, we observed that depletion of B cells in the ID8 ovarian mouse model decreased the expression of the degranulation marker, CD107, on CD8⁺ T cells. This was accompanied by increased tumor growth and suggested that depletion of B cells impairs the T cell dependent anti-tumor cytotoxic response. In accordance with these results, RNAseq analyses of human omental

metastases showed CD20 transcript levels significantly correlated with levels of cytolytic markers (22), hence linking B cells to immune cytotoxic responses in patients' metastases.

It is interesting to note that chemotherapy neither affected the enrichment in B cell signature nor the density of CD20⁺ B cells, however, class-switched memory B cells were enhanced after NACT. Although we cannot rule out a direct effect of chemotherapy on survival and migration of B cells, it is possible to speculate that following chemotherapy the release of tumor antigens in the microenvironment provides a boost for the activation and differentiation of B cells. Nonetheless, these results are in accordance with studies describing the immune-stimulatory properties of chemotherapeutic agents such as anthracyclins, which causes immunogenic cell death of tumor cells (28) and platinum-based chemotherapy, shown to promote T cell activation and downregulation of immune-modulatory molecules on DCs (29). Our results indicate that chemotherapy has a positive influence also on B cell responses.

Next, we observed that omental B cells produced a network of cytokines and chemokines including IFN γ , IL12, GM-CSF and CXCL10, compatible with the development of anti-tumor responses, most likely occurring within TLS. HGSOC omental B cells also secreted high amounts of CXCL8, a chemoattractant for neutrophils and DCs. Indeed, CXCR1 and CXCR2 receptors for CXCL8 were detected on omental DCs and neutrophils. Although neutrophils expressed higher levels of both receptors, more DCs than neutrophils infiltrated the lymphoid structures found in omental metastases. A more complex chemotactic network comprising CCL2, CXCL8, GM-CSF but also additional unidentified factors might explain this observation. Interestingly and in accordance with a study conducted in NSCLC (8), stromal densities of CD20⁺ B cells and DC-LAMP⁺ DCs correlated in omental

tumors. *In vitro* studies recently demonstrated that activated B cells could induce the maturation of monocyte-derived DCs, rendering them competent for T cell activation (30). Whether omental B cells directly support maturation of DCs in a similar fashion remains to be determined. Nonetheless, the spatial distribution of B cells and DCs within the TLS and B cell cytokine and chemokine production suggest that B cells promote not only DC recruitment but also their differentiation. Are B cells also involved in increasing DC antigen-presenting ability? In this respect, we observed a high deposition of IgGs in the tumor stroma, some of which were bound to APCs. Several factors might contribute to this stromal localization, (i) the extracellular matrix could impair the diffusion of IgGs, preventing them from reaching tumor cells, (ii) ongoing cell death due to necrosis, chemotherapy and/or immunity, which favors the release of antigens in the microenvironment, hence sequestering antigen-specific IgGs in the stroma, (iii) IgGs targeting intracellular antigens that cannot be easily accessible. We confirmed that intratumor IgGs preferentially target intracellular antigens, although some cytoplasmic proteins can be abnormally exposed at the surface of cancer cells (31). Nonetheless, previous studies provided evidence that Igs targeting intracellular antigens possess anti-cancer properties either by reaching their targets, possibly through internalization by cancer cells (32,33) or by activating APCs through FcR, which in turn stimulate T cell responses (34). In support of this latest hypothesis, we found that immunoglobulins from HGSOc metastases can form ICs with tumor antigens and promote expression of the co-activatory molecule CD86 on *in vitro* generated DCs. These results indicate that ICs present in the tumor stroma could increase DC antigen-presenting capabilities.

Contrary to studies showing an increase in IgG4 antibodies in cutaneous metastases of melanoma patients (35), the predominance of IgG1, IgG2 and IgG3 over IgG4

antibodies in HGSOC metastases, especially following chemotherapy treatment, highlighted their potential to trigger APCs activation, through binding to activatory Fc γ Rs (36,37). It is interesting to note that in post-chemotherapy omentum displaying low residual disease the decrease in tumor IgGs, mirrored by the decrease in the B cell infiltrate in the tumor, is likely to be linked to a partial resolution of the anti-tumor immune response with a decreased number of TLS.

In conclusion, our study demonstrated for the first time that in human metastases of HGSOC, B cells develop memory responses in the tumor microenvironment, likely via their association with TLS. In this context, their activated phenotype, secretion of pro-immunogenic cytokine/chemokines and production of IgGs targeting tumor antigens is associated with an enhanced recruitment and priming of DCs and promotion of immune cytotoxic responses. Our study provides supporting evidence that ICs of anti-tumor IgGs bound to tumor antigens could be beneficial in the clinic and potentiate anti-tumor responses. This approach has already been tested in a pre-clinical study (23) and should be further explored in a cancer setting. Since we show that chemotherapy further enhances the anti-tumor B cell response, these new approaches would be compatible and aided by current treatments.

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Table.

Table 1. Identification of the proteins targeted by immunoglobulins in HGSOC omentum by mass spectrometry.

	IgG Targets	N° of unique peptides	Mascot score	Enrichment compared to total extract		
				G175	G168	G146
Pulled down from both G168 and G175 omentum	Guanine nucleotide-binding protein subunit alpha-13 OS=Homo sapiens GN=GNA13 PE=1 SV=2	1.0	92.8	1750.9	550.3	
	Protein 4.1 OS=Homo sapiens GN=EPB41 PE=1 SV=4	2.0	92.6	14.7	2.8	
Pulled down from G175 omentum only	Nexilin OS=Homo sapiens GN=NEXN PE=1 SV=1	3.0	78.3	177.4		
	60S acidic ribosomal protein P0 OS=Homo sapiens GN=RPLP0 PE=1 SV=1	3.0	188.8	11.7		
	Myosin light chain 6B OS=Homo sapiens GN=MYL6B PE=1 SV=1	3.0	146.1	25.2		
	CD5 antigen-like OS=Homo sapiens GN=CD5L PE=1 SV=1	1.0	83.7	26.6		
	Dihydropyrimidinase-related protein 2 OS=Homo sapiens GN=DPYSL2 PE=1 SV=1	4.0	180.5	2.6		
	60S ribosomal protein L6 OS=Homo sapiens GN=RPL6 PE=1 SV=3	4.0	153.6	3.8		
	Actin, aortic smooth muscle OS=Homo sapiens GN=ACTA2 PE=1 SV=1	2.0	962.8	5.6		
	Cell surface glycoprotein MUC18 OS=Homo sapiens GN=MCAM PE=1 SV=2	2.0	79.2	3.3		
	78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2	8.0	370.0	2.7		
	Filamin-C OS=Homo sapiens GN=FLNC PE=1 SV=3	3.0	164.4	7.6		
	Dihydropyrimidinase-related protein 3 OS=Homo sapiens GN=DPYSL3 PE=1 SV=1	5.0	321.9	15.9		
	Inter-alpha-trypsin inhibitor heavy chain H1 OS=Homo sapiens GN=ITIH1 PE=1 SV=3	2.0	130.2	3.2		
	Interferon-induced GTP-binding protein Mx2 OS=Homo sapiens GN=MX2 PE=1 SV=1	2.0	218.2	64.0		
	Inter-alpha-trypsin inhibitor heavy chain H4 OS=Homo sapiens GN=ITIH4 PE=1 SV=4	4.0	164.4	4.2		
	AP-2 complex subunit alpha-1 OS=Homo sapiens GN=AP2A1 PE=1 SV=3	2.0	186.0	2.9		
	Keratin, type II cytoskeletal 8 OS=Homo sapiens GN=KRT8 PE=1 SV=7	1.0	137.5	2.7		
	40S ribosomal protein S20 OS=Homo sapiens GN=RPS20 PE=1 SV=1	2.0	103.9	3.2		
	Tripeptidyl-peptidase 1 OS=Homo sapiens GN=TPP1 PE=1 SV=2	1.0	83.1	4.4		
	Calreticulin OS=Homo sapiens GN=CALR PE=1 SV=1	2.0	90.6	8.9		
Pulled down from G168 omentum only	Hemoglobin subunit gamma-1 OS=Homo sapiens GN=HBG1 PE=1 SV=2	5.0	370.3		7.2	
	Heat shock protein beta-1 OS=Homo sapiens GN=HSPB1 PE=1 SV=2	5.0	115.5		5.0	
	Histidine-rich glycoprotein OS=Homo sapiens GN=HRG PE=1 SV=1	2.0	101.9		3.6	
	Alpha-actinin-2 OS=Homo sapiens GN=ACTN2 PE=1 SV=1	1.0	470.3		2.6	
Pulled down from G146 omentum only	Desmin OS=Homo sapiens GN=DES PE=1 SV=3	1.0	101.8			31.8
	POTE ankyrin domain family member E OS=Homo sapiens GN=POTEE PE=1 SV=3	1.0	330.3			514.6

Table and Figure legend:

Table 1. Identification of the proteins targeted by immunoglobulins in HGSOC omentum by mass spectrometry. Tumor IgGs were used as bait to immunoprecipitate their potential targeting antigens. Proteins found significantly enriched in the IP fractions compared to total tumor lysate were considered potential targets of tumor IgGs.

Figure 1. B cells in HGSOC are found in tertiary lymphoid structures. (A) Immunohistochemistry pictures showing two representative sections from paraffin embedded HGSOC omentum stained for CD20. Scale bar: 200µm. (B and C) Log₂ density of CD20⁺ B cells in HGSOC omental metastases. (B) Comparison of CD20⁺ B cell densities in tumor vs stromal areas (n=33). (***)p ≤ 0.001; Mann-Whitney test) (C) Comparison of CD20⁺ B cell densities in the stroma of unmatched HGSOC metastases obtained before (n=10) and after chemotherapy treatment (n=31). (D-F) Characterization of the B cell-rich lymphoid structures by single color immunohistochemistry, immunofluorescence and double color immunohistochemistry. (D) Identification of CD20⁺ B cells, CD4⁺ and CD8⁺ T cells within TLS. Scale bars: 50µm. (E) Presence of MECA79⁺ high endothelial venules in TLS. Scale bars: 20µm. (F) Germinal centers of TLS contain follicular dendritic cells (FDC) and Ki67⁺ high proliferating cells surrounded by CD45RO⁺ memory cells. Scale bars: 50µm.

Figure 2. Memory B cell response in HGSOC metastases. (A and B) Flow cytometry analysis of the B subpopulations in HGSOC omentum (n=25) showing differential expression of CD27 and IgM within the CD20⁺ cells. Representative plots (A) and percentages (B) of the different B cell subpopulations: IgM⁺ memory

(CD27⁺IgM⁺), class switched memory or IgM⁻ memory (CD27⁺IgM⁻), CD27⁻ atypical memory (CD27⁻IgM⁻) and naïve (CD27⁻IgM⁺) B cells. An example of IgD expression in the different subpopulations is shown. (C and D) Proportion of CD86⁺PD1⁻ (C) and PD1⁺CD86⁻ (D) cells among the B cell subsets described above (n=11). (*p≤0.05; **p≤0.01; ***p≤0.001; Mann-Whitney test and Student's T test). (E and F) BCR sequencing of B cells from HGSOC omentum (O) (n=7) and blood (B) (n=1) compared to healthy peripheral B cells (ctrl) (n=7). (E) Diversity analysis of the B cell repertoire. Error bars: 95% confidence interval (38). (F) Analysis of hypersomatic mutations. *IgH* mutation rates for each sample (top panel). Tumor and non-tumor samples were binned and *IgHV* mutation rates compared (lower panel).

Figure 3. Role of B cells in the recruitment of dendritic cells in HGSOC metastases. (A-D) Cytokine/chemokine secretion of HGSOC omental B cells (see supplementary Methods). (A) Correlation heatmap showing how the levels of the 24 cytokines and chemokines measured in the supernatant of HGSOC B cells (n=4-10) vary with each other. Columns and rows represent individual cytokines and chemokines colored to indicate pearson's correlation coefficient (r). (B-D) Log₂ concentrations of CXCL8, CCL2 and IL6 in the supernatant of unstimulated healthy peripheral B cells (n=2); PMA/Ionomycin (PMA/Iono) treated healthy peripheral B cells (n=5-7); unstimulated and PMA/Ionomycin treated HGSOC omental B cells (n=9-10). (*p≤0.05; ***p≤0.001, Student's T test) (E) Flow cytometry analysis of CXCR1 (n=4) and CXCR2 (n=3) expression on CD19⁺ B cells; CD3⁺ T cells, CD11b⁺⁺CD14⁺CD11c⁻ macrophages; CD11b⁺⁺CD14⁻CD11c⁻ granulocytes and CD11b⁺⁺CD14⁻CD11c⁺ dendritic cells from HGSOC omentum. (F) Representative immunostainings and quantification of DC-LAMP⁺ dendritic cells and MPO⁺

neutrophils in CD20⁺ rich lymphoid structures in HGSOC omental metastases (n=7). Arrows indicate MPO⁺ cells. Scale bar: 100μm. (***p≤0.001, Student's T test) (G) Correlation analysis between the log₂ densities of DC-LAMP⁺ dendritic cells and CD20⁺ B cells in a cohort of 16 HGSOC patients (2 pre-chemotherapy and 14 post-chemotherapy) (p≤0.05; Spearman correlation test).

Figure 4. B cell signature is enriched in diseased omentum and linked to cytotoxic response. (A-D) Heatmap and boxplots showing an increase in expression of B cell signature genes in stage 3/4 pre (n=4) and post-chemotherapy (post-chemotherapy diseased (post dis) n=7; post-chemotherapy good responders (post good) n=5) vs stage 1/2 (n=6) non-involved HGSOC omentum. (*p≤0.05; **p≤0.01; Student's T test). (E) Positive correlation between the geometrical mean of levels of expression of granzyme A (*GZMA*) and perforin (*PRFI*) versus CD20 (*MS4A1*) in HGSOC stage 3/4 omentum. (F) CD20 depletion increased tumor progression in the ID8 mouse model. (Left panel) Tumor progression in anti-CD20 (n=5) and IgG control (n=4) treated mice assessed by luciferase activity monitoring (p=0.03; Two-way ANOVA on Log₁₀ luminescence values). (Upper right panel) B cell (CD45⁺ CD19⁺) and plasma cell (CD45⁺ CD138⁺) are depleted in the ascitic fluid of αCD20 treated ID8 mice vs IgG controls. (Lower right panel) CD107 expression by ascitic CD8⁺ T cells in αCD20 and IgG control treated ID8 mice (**p≤0.001; Student's T test).

Figure 5. Plasma cells are present in HGSOC omenta and IgGs deposited mainly in stromal areas but also on tumor cells and macrophages. (A and B) Presence of CD38⁺⁺CD19^{+/-}CD20⁻CD27⁺ plasma cells in HGSOC omentum (n=11). (C) Paraffin

sections of omental metastases were fluorescently stained for CD20, CD68 or Cytokeratin 7 (CK7) (red) in combination with IgG (green). Arrows indicate co-localization of the CD68 and CK7 markers with IgG. Scale bar: 20µm. (D) Protein extracts isolated from frozen stage 3/4 pre-chemotherapy, post-chemotherapy diseased, post-chemotherapy with no/low residual disease and Stage 1/2 non involved omentum were tested for the presence of human IgG1, IgG2, IgG3 and IgG4 by ELISA. (* $p < 0.05$, Mann Whitney). (E) Tumor derived IgGs ICs increase the expression of CD86 on DCs *in vitro*. IgGs were isolated from two tumor lysates using Agarose beads coupled to protein A/G and pooled. Monocyte-derived DCs (n=7) from healthy volunteers were then treated with tumor IgGs alone or tumor IgGs pre-incubated with protein extracts from HGSOc tumor cells. IgGs from healthy plasma was used as a control. CD86 expression on DCs was assessed after 24h treatment.

Figure 1

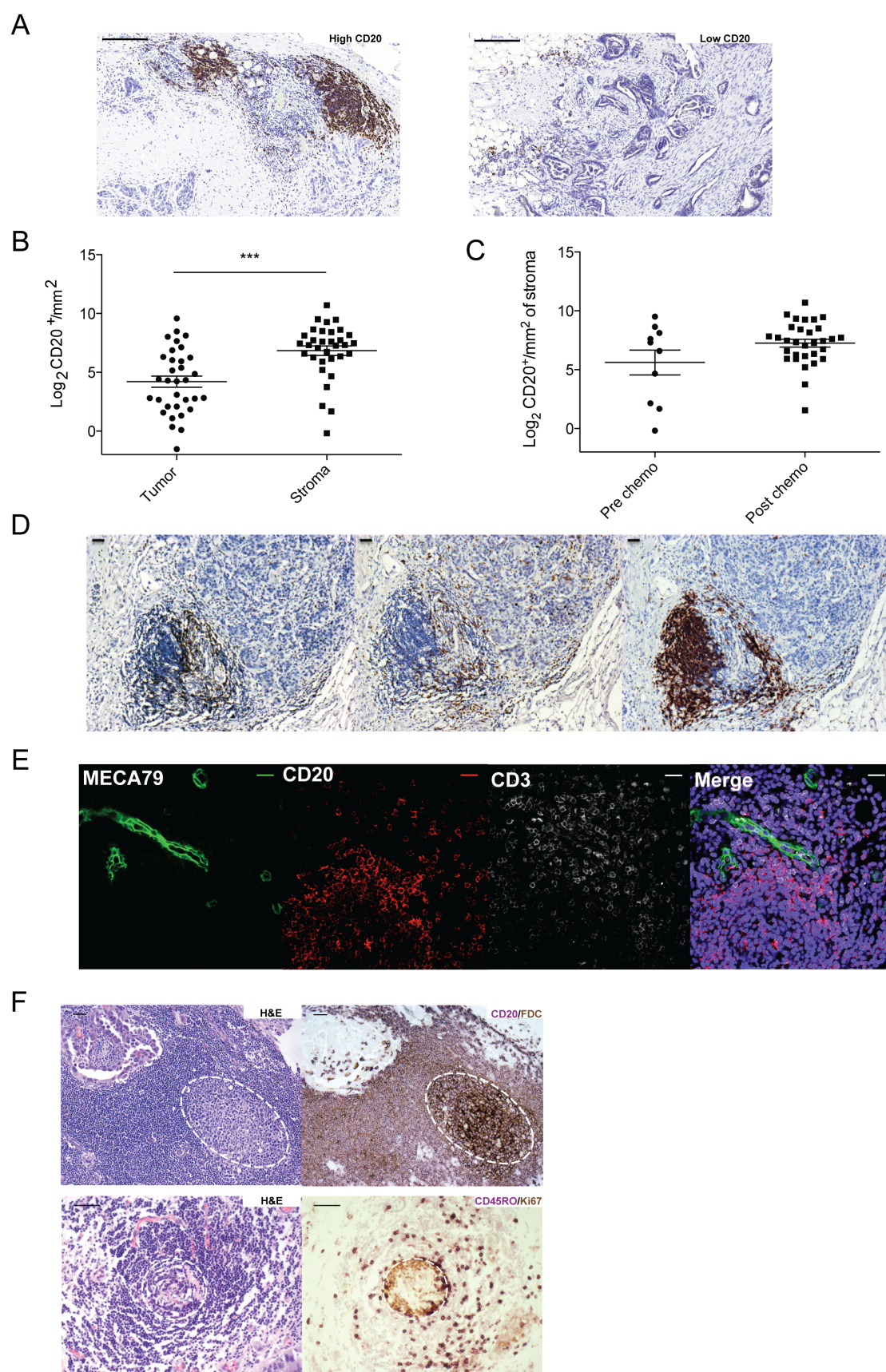


Figure 2

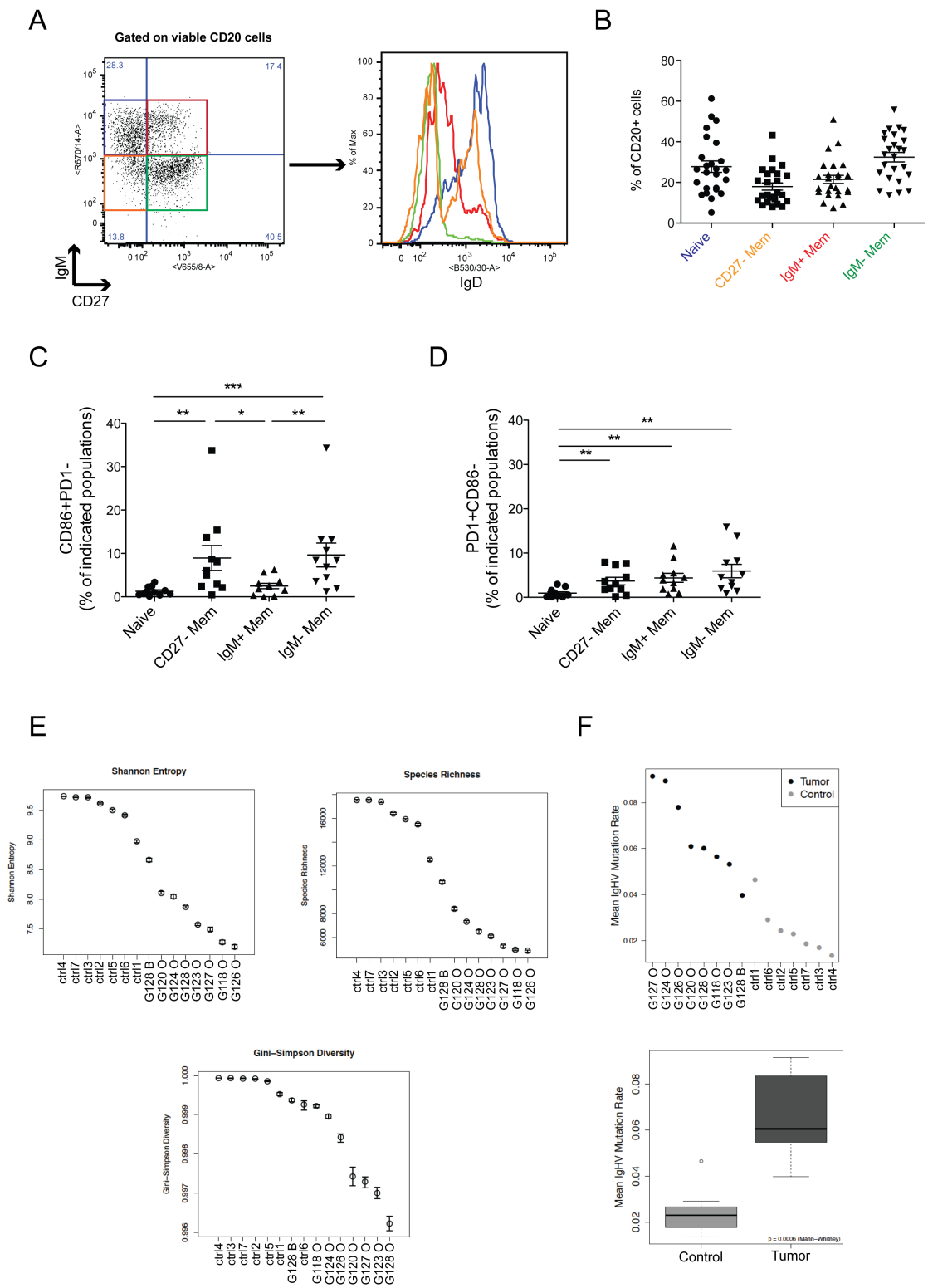


Figure 3

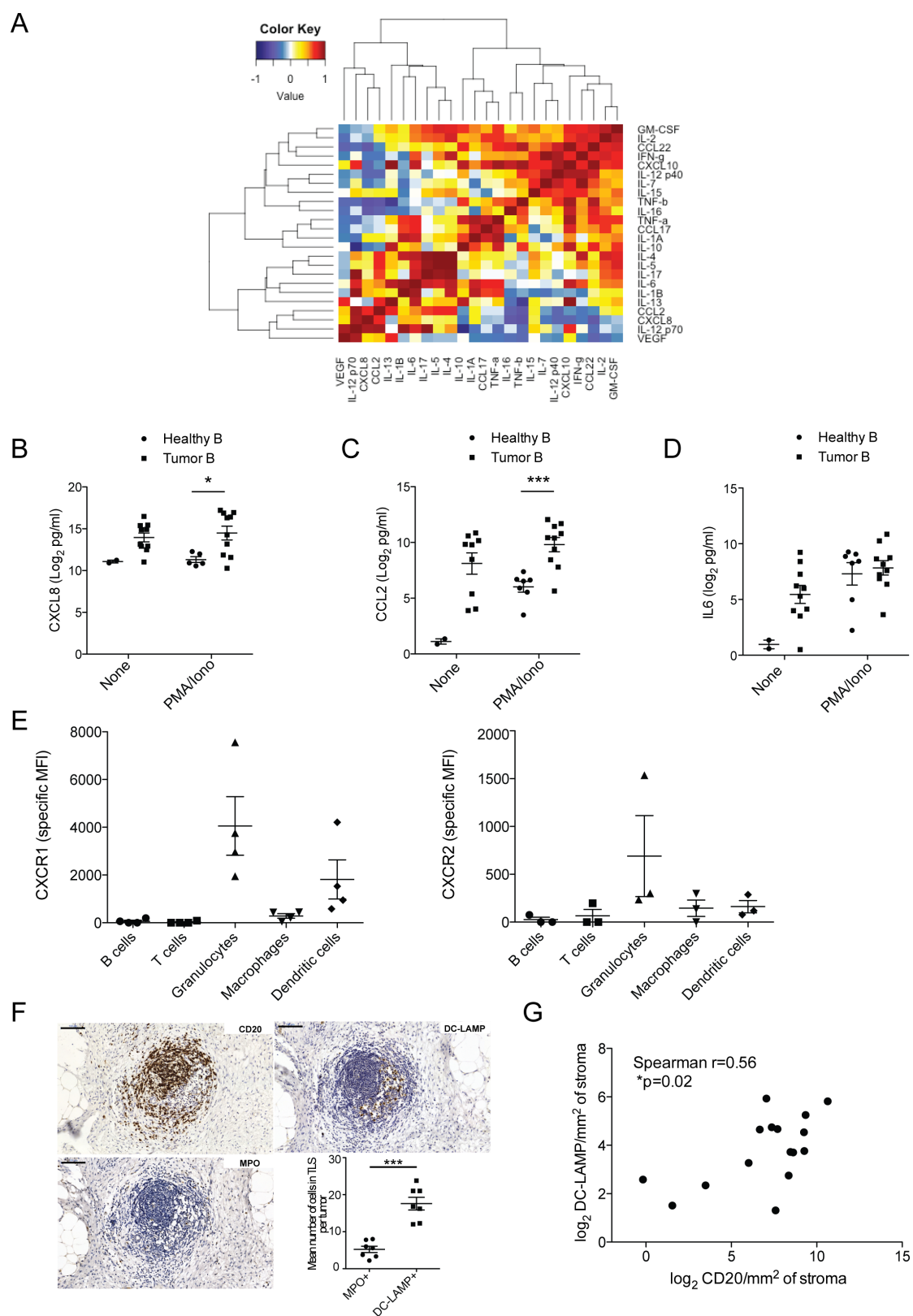


Figure 4

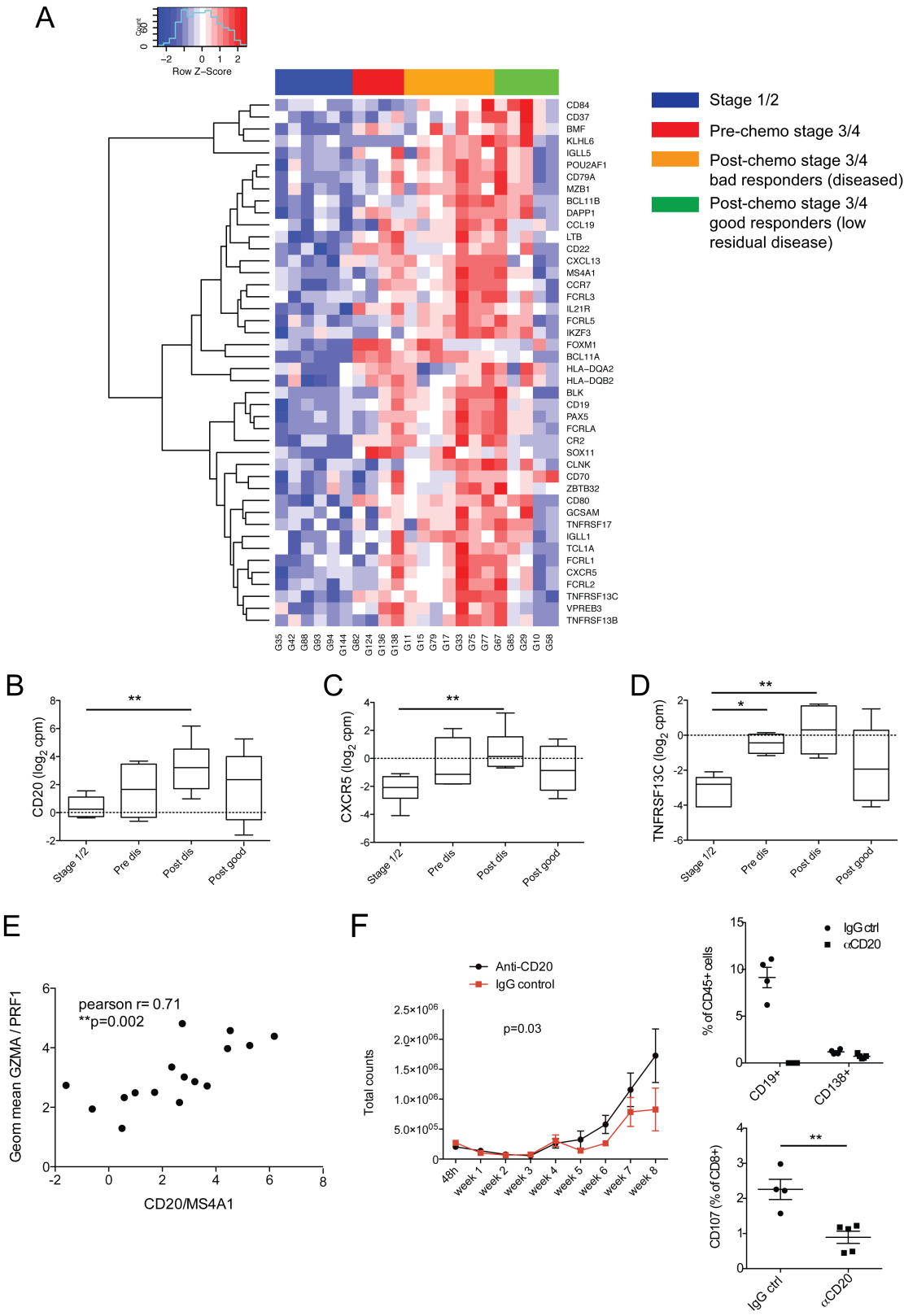


Figure 5

